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(57) Abstract

A process for preserving a material having water-dependant structure comprising contacting the material with an aqueous solution of a polyhydroxy compound then removing water from the material.

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PROTEIN PRESERVATION

The present invention relates to a method of dry storage which now permits the long-term preservation of materials such as haemoglobin, erythrocyes, liposomes and cells.

Oxidation of the iron atom of the haem prosthetic group from 2+ to the 3+ state converts oxy- or deoxyhaemoglobin to the biologically nonfunctional methaemoglobin. The reaction is promoted by conditions of low oxygen tension and is minimised in vivo by the presence of potent reducing systems operating in the cytoplasm of intact erythrocytes. When erythrocytes are obtained from human donors for transfusion, their storage ex vivo is limited, in part, by the accelerated formation of oxidised haem (methaemoglobin).

Storage in the dry state limits hydrolysis and prevents some of the oxidative damage that can destroy chemical or biological activity. When dealing with biological macromolecules, however, water removal can irreversibly alter secondary, tertiary or quaternary structures and thereby eliminate biological efficacies.

Dehydration of biological membranes is usually accompanied by irrevocable loss of structural and functional integrity (Crowe, Crowe and Jackson, 1983). Upon rehydration of biological membranes morphological

damage is apparent in the redistribution of intramembraneous particles and the fusion between membranes. Moreover, in those membrane systems that are associated with a specific enzyme activity (such as the transport of Ca^{++}), rehydration induces a complete loss of activity.

Artificial membrane systems prepared by dispersion of phospholipids in aqueous solutions, have received much attention because of their utility in basic research and their potential applications in biotechnology. Dispersions of phospholipids spontaneously form sealed structures called liposomes - the encapsulated volume within a liposome enables the liposome to function as a pharmacological "capsule" or artificial cell. Among the solutes that have been encapsulated in liposomes is human haemoglobin. The "haemosomes" so generated hold much utilitarian promise as erythrocyte surrogates (Djordjevich and Miller, 1980; Gaber et al., 1983; Hayward et al., 1985). However when liposomes containing entrapped solutes are subjected to dehydration/rehydration, the solutes are lost to the bulk solution (Madden et al., 1985).

The effect of encapsulation of haemoglobin within a phospholipid liposome was to catalyze the oxidation of haemoglobin upon rehydration. No oxyhaemoglobin could be regenerated from rehydrated haemosomes. Moreover, much of the visible absorbance of the rehydrated samples was bleached, resulting in essentially colourless samples.

These observations are similar to those described by Szebeni et al. (1985), who found that unsaturated phospholipids promoted haemoglobin oxidation and were capable of extracting the haem away from the globin portion of the molecule.

We have found that by drying proteins from aqueous solutions containing saccharides, chemical and physical degradative processes can be avoided. If the carbohydrate selected is itself a solid, then a compact, solid protein-carbohydrate mixture is obtained which can be repeatedly cooled below 0°C and warmed to room temperature without diminishing enzyme activity. Moreover this technique may be extended to facilitate storage of other materials having water-dependant structure such as liposomes and even intact cells.

We have examined the ability of trehalose and other sugars to preserve after dehydration/rehydration the biological activity of isolated proteins, protein systems, biological and artificial membranes, and whole cells.

The present invention therefore provides a process for preserving a material having a water-dependant structure comprising contacting the material with an aqueous solution of a polyhydroxy compound then removing water from the material.

By "material having a water-dependant structure" it is intended to encompass such materials as proteins, liposomes and cells which have secondary, tertiary or

quaternary structure determined by hydrophobic and/or hydrophilic interactions in the presence of water which structure is irreversibly altered or partially or completely destroyed by removal of the water.

By "polyhydroxy compound" it is intended to encompass any material having two or more hydroxy groups per molecule which is capable of hydrogen bonding to, or otherwise stabilising, a material having a water-dependant structure. Suitable polyhydroxy compounds include sugars and similar or related polyols, especially trioses, pentoses and hexoses, mono-, di or oligosaccharides and related polyols. Preferred polyhydroxy compounds are glucose, galactose and non-reducing sugars especially trehalose.

The present invention, in a particular embodiment also provides a process for preserving a material having a water-dependant structure which encapsulates one or more spaces containing aqueous solutions or suspensions which comprises contacting the material with, and introducing into one or more encapsulated spaces therein, an aqueous solution of polyhydroxy compound then removing water from the material.

The present invention in another aspect provides a dry formulation comprising a material having a water-dependant structure and a polyhydroxy compound. The polyhydroxy compound will be in intimate admixture with the material having a water-dependant structure and, in the

case where the material encapsulates one or more spaces containing the residue of aqueous solutions or suspensions, preferably the polyhydroxy compound is also dispersed within those spaces.

The preservation process of the present invention is particularly suitable for the storage of haemoglobin with minimal oxidation of the heme-iron and with retention of gas-transporting capacity upon rehydration. The dry haemoglobin may be prepared from simple aqueous solutions of purified haemoglobin, from erythrocyte lysates, from intact erythrocytes or from erythrocyte surrogates (haemosomes).

Whilst not wishing to be bound by any theory it is postulated that the stabilisation afforded according to the present invention may be related to that observed in certain instances of "anhydrobiosis".

The organisation of many biological structures depends absolutely upon the presence of water. Hydrophobic structures, such as the membranes which delimit all cells and their organelles, arise by the expulsion of water from their non-polar parts. Hydrophilic structures such as globular proteins are stabilised by hydrogen-bonding with water in solution and by the maintenance of specific dipoles in a medium of specific dielectric constant.

Some organisms, however, possess a remarkable capacity to survive near-total dehydration, and they may exist in the dehydrated states for periods measured in

years. This fact was recognised as long ago as the year 1800 when it was found that in a dried state tardigrades, small sand-living organisms, were able to withstand temperatures well below freezing and as hot as boiling water (Crowe and Clegg, 1973 and 1978). Normal metabolic processess resumed in these organisms within thirty minutes of their rehydration even after decades of dry storage.

In recent years the ability to survive desiccation, now termed "anhydrobiosis", has been shown to be a property of a small but diverse group of organisms including fungal spores, macrocysts of the slime mold Dictyostelium, baker's yeast, brine shrimp cysts, nematodes and plant seeds (for review see Crowe and Crowe, 1984).

Trehalose, a non-reducing disaccharide of glucose that is distributed widely in nature, has been found at particularly high concentrations (as much as 20% of the dry weight) in several of the organisms capable of anhydrobiosis. The survival of dehydration by some organisms is correlated with the synthesis of trehalose during dehydration (Madin and Crowe, 1975) or its degradation following rehydration (Clegg. 1964). This molecule was implicated in the "water replacement hypothesis", which suggested that the hydroxyl groups of simple carbohydrates might replace the hydration shell of the polar head groups of phosopholipids that is lost during dehydration (Crowe and Clegg, 1973).

Recent experiments using infrared spectroscopy

(Crowe, Crowe and Chapman, 1984), have provided evidence that trehalose may substitute for water by forming hydrogen bonds with the head groups of phospholipid bilayers thereby stabilising membrane structure in the absence of water. Support for this model was obtained by Madden et al. (1985) who demonstrated that the integrity of unilamellar liposomes is retained (i.e. no leakage of solute from the internal aqueous compartment of the liposome) if dehydration is carried out in the presence of carbohydrate. This protective capacity was shared among all of the carbohydrates tested.

Sugars and similar poly alcohols have been utilised in solution to preserve enzymatic activities (Bradbury and Jakoby, 1972), inhibit irreversible aggregation (Frigon and Lee, 1972) and increase the temperature at which proteins are thermally denatured (Gerlsma, 1968; Neucere and St. Angelo, 1972).

Due to the widespread use of proteins in analytical, synthetic and biological chemistry, we envisage that carbohydrate-mediated protection of dried proteins will have many applications. The storage stability of most proteins should be enhanced by the processes we describe.

A particularly novel extension of our invention concerns the stabilisation of dehydrated cells and their surrogates. It is possible to transiently produce pores in both biological and artificial membrane which spontaneously reseal. During the time course of their existence,

however, these pores are of sufficient diameter to permit the entry of carbohydrates into the cytoplasm (cells) or encapsulated volume (liposomes). Thus, it should be possible to introduce trehalose (or other carbohydrates) into the cytoplasm of human erythrocytes, enabling their extended storage in the dehydrated state. Autologous red cells could therefore be stored indefinitely for patients at high risk of anaemia. Similarly, haemosomes could be prepared and stored in bulk following dehydration. Application of dry haemosomes would be a simple matter following their rehydration ad hoc.

The invention will now be illustrated by non-limiting Examples and with reference to the accompanying drawings in which; Fig. 1 is a histogram showing Percentages of methaemoglobin formed upon rehydration of haemoglobin powders. Sugars were present at concentrations of 0.25 molar following rehydration; haemoglobin was 0.015 molar. 1) Oxyhaemoglobin without sugar, 2) Deoxyhaemoglobin without sugar, and 3) to 11) Oxyhaemoglobin with 3) Arabinose, 4) Glactose, 5) Fucose, 6) Glucose, 7) Mannose, 8) Maltose, 9) lactose, 10) Trehalose and 11) Sucrose.

EXAMPLES 1 AND 2

Different sugars are utilised to produce dry powders of two separate proteins, chicken egg-white

lysozyme (Example 1) and human haemoglobin (Example 2). Structural stability was examined by calorimetry of the wet and dry lysozyme preparations. Functional stability was verified by spectroscopic evaluation of gas transport by haemoglobin.

Carbohydrate-protein mixtures were prepared in solution by addition of carbohydrate to the protein dissolved in water or buffer. A variety of trioses, pentoses and hexoses were examined as well as mono- and disaccharides. Concentrations of the sugars were varied, from 1:1 (weight:weight) up to a maximum of 0.25 molar sugar.

Water was removed by one of three methods: a) evaporation, in which the solution is mildly heated under a steady stream of gaseous nitrogen; b) evacuation, in which the sample is exposed to reduced pressure at room temperature, or, c) lyophilisation, in which the solution is first frozen in liquid nitrogen and the water sublimed under high vacuum. Dried samples were stored for extended period over phosphorus pentoxide in vacuo.

Calorimetric evaluation was performed by differential scanning calorimetry (DSC) using Perkin-Elmer DSC-2. The heating rate was 2.5°C/minute and a full-scale deflection on the recorder corresponded to a change in heat flow of 0.25m.cal./sec. The transition temperatures obtained were the temperatures of maximal heat flow.

Table 1 contains the denaturation temperatures

and denaturation enthalpy and entropy changes of lysozyme dissolved in water and in solutions of either of the sugars glucose, galactose or trehalose. The presence of any of the three sugars increases the denaturation temperature, in general agreement with the observations reported by others (Gerlsma, 1968; Neucere and St. Angelo, 1972). Hence, in solution, the sugars stabilise the native structure of the protein with respect to the denatured state. This property is exhibited by each of the three sugars examined.

TABLE ONE

DENATURATION TEMPERATURES AND DENATURATION ETHALPY AND ENTROPY CHANGES OF LYSOZYME IN PURE WATER AND AQUEOUS SUGAR SOLUTIONS

	Concn. Lysozyme in Water (w/w%)	Sugar	Ratio Sugar to Lysozyme (mole:mole)	T_d (°C)	ΔH_{cal} (kcals.-mole)	ΔS_{cal} (cals.-kmole)
a	5	None	-	72.8	142	410.5
b	10	"	"	70.9	116.2	337.7
c	20	"	"	68.8	105.7	309.1
d	10	Glucose	1:1	73.8	118.9	342.7
e	"	Trehalose	"	73.6	119.6	344.9
f	"	Galactose	"	73.5	139.0	401.0
g	"	"	"	73.6	133.0	384.2
h	5	"	"	73.9	136.3	392.7
i	20	"	"	75.3	101.0	289.6
j	10	Glucose	5:1	83.2	88.8	249.8
k	"	Trehalose	"	79.8	111.5	315.8
l	"	Galactose	"	81.5	90.4	254.9

The temperatures of maximum heat flow and the enthalpy and entropy changes associated with the transitions for freeze-dried lysozyme in the presence or absence of carbohydrate are outlined in Table 2. The endothermic transition of pure dry lysozyme occurs at a temperature approximately 70°C above the denaturation temperature of the protein in water. In the solid state the polypeptide chains of a protein will be less flexible than in solution at the same temperature.

Therefore, a dry protein will reach the degree of flexibility necessary for unfolding of the protein structure at a higher temperature than a protein in aqueous solution.

TABLE TWO

TRANSITION TEMPERATURES AND TRANSITION ENTHALPY AND ENTROPY CHANGES OF FREEZE DRIED LYSOZYME AND SUGAR-LYSOZYME MIXTURES

	Ratio Sugar to Lysozyme (mole:mole)	Sugar	Mean T_{tr} (°C)	S.D./ Range	Mean ΔH_{cal} (kcals.-mole)	S.D./ Range	ΔS_{cal} (cals.-kmole)	number of tests
a	1:1	Glucose	117.0	3.56	152.6	13.1	391.1	8
b	"	Trehalose	127.3	5.56	123.8	7.6	309.2	11
c	"	Galactose	128.1*	3.25	368.0	22.1	917.2	6
			110.9	3.45	66.0	4.1	171.9	
d	5:1	Glucose	122.5	123.7-120.9	348.3	351.7-344.9	880.3	3
e	"	Trehalose	139.4	1.65	140.1	13.9	339.6	4
f	Pure lysozyme		139.6	5.80	63.9	13.9	154.8	9
g	Pure lysozyme (freeze dried)		128.8	9.80	49.8	11.1	123.9	8

X Thermogram displays two separate endotherms

In the presence of sugar, the transition temperature is decreased, and the enthalpy and entropy changes for the transition equal or exceed those values seen for the protein in solution. Thus, it is apparent that as the water of hydration is removed from the surface of the protein, it is gradually replaced by sugar, resulting in a stabilisation of protein conformation that does not occur in the absence of added sugar.

Although each of the three sugars studied was capable of restoring the calorimetric behaviour of dissolved lysozyme to the dried protein, it was observed that the heated powders containing glucose or galactose were "browned". This browning reaction did not occur in the presence of trehalose. Thus, the selective pressure toward the occurrence of trehalose in anhydrobiotic organisms may be due less to its capcity to substitute for water than to its non-reducing characteristics which prevent the browning reaction. Nonetheless, in our invention any of the above-mentioned sugars are appropriate for storage of dry protein-sugar mixtures.

These results were extended by determinations of the functional integrity of a human haemoglobin before and after dehydration/rehydration in the presence or absence of various carbohydrates. The relative concentrations of oxy-, deoxy- and methaemoglobins were calculated from the spectra obtained in solution according to the methods described in Benesch et al. (1965).

In Figure 1 are presented the percentages of methaemoglobin formed in solutions of human haemoglobin following a cycle of dehydration/rehydration. When dried haemoglobin without sugar was rehydrated, the solution assumed the brown colour characteristic of methaemoglobin. The percentage of methaemoglobin formed increased with the duration of storage, up to 90% of the total haem. Similar spectra could be generated by catalytic oxidation of the haem after addition of potassium ferricyanide.

In strong contrast to the behaviour of rehydrated haemoglobin in the absence of sugar, each of the carbohydrates tested was capable of protecting haemoglobin against oxidative damage. The protective effect was clearly manifest even in those samples that had been stored in the dried state for periods in excess of three months. Rehydrated sugar-haemoglobin mixtures were capable of reversible gas exchange as evidenced by the spectral shifts induced by exposure to oxygen, nitrogen or carbon monoxide.

Together, our results obtained with dried powders of lysozyme or haemoglobin demonstrate that carbohydrates enhance the stability of anhydrous proteins and permit the retention of physicochemical and biological activities after rehydration.

EXAMPLE 3

Our previous investigations of hemosomes as erythrocyte surrogates (Hayward et al., 1985) in

combination with our demonstration of protein stabilisation in the dry state by carbohydrate, prompted us to examine the capacity of sugars to stabilize artificial red blood cells in the dry state. We reasoned that the protective capacity of carbohydrate in this instance should be bimodal - prevention of the oxidation and denaturation of haemoglobin and the simultaneous retention of encapsulated haemoglobin within the internal aqueous compartment of the haemosome.

We found that inclusion of carbohydrates (both within the haemosome and in the external, bulk solution) prevented haem oxidation. Furthermore, rehydrated carbohydrate/haemosomes could be passed through a molecular sieve without diminution in the ratio of haemoglobin:phospholipid. All of the carbohydrates tested to-date have been effective in this capacity. Conversely, carbohydrate-free haemosomes became irreversibly aggregated and the phospholipid was readily separated from haemoglobin due to the former's relative buoyancy.

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CLAIMS

1. A process for preserving a material having water-dependant structure comprising contacting the material with an aqueous solution of a polyhydroxy compound then removing water from the material.

2. A process according to claim 1 wherein the material is a protein, liposome or cell.

3. A process according to claim 1 wherein the material is an erythrocyte, oxyhaemaglobin, deoxyhaemaglobin or liposomes encapsulating oxyhaemaglobin or deoxyhaemaglonin.

4. A process according to claim 1 wherein the material encapsulates one or more spaces containing aqueous solutions or suspensions and wherein the process comprises contacting the material with, and introducing into one of more encapsulated spaces therein, an aqueous solution of a polyhydroxy compound then removing water from the material.

5. A process according to claim 1 wherein the polyhydroxy compound is a sugar or related polyol.

6. A process according to claim 5 wherein the polyhydroxy compound is selected from trioses, pentoses, hexoses, mono-, di- or oligosaccharides and related polyols.

7. A process according to claim 6 wherein the polyhydroxy compound is selected from glucose, galactose and non-reducing sugars.

8. A process according to claim 7 wherein the polyhydroxy compound is trehalose.

9. A dry formulation comprising a material having a water-dependant structure and a polyhydroxy compound in intimate admixture therewith.

10. A formulation according to claim 9 wherein the material is a protein, liposome or cell.

11. A formulation according to claim 9 wherein the material is an erythrocyte, oxyhaemaglobin, deoxyhaemaglobin or liposomes encapsulating oxyhaemaglobin or deoxyhaemaglobin.

12. A formulation according to claim 9 wherein the material encapsulates one or more spaces containing aqueous solutions or suspensions.

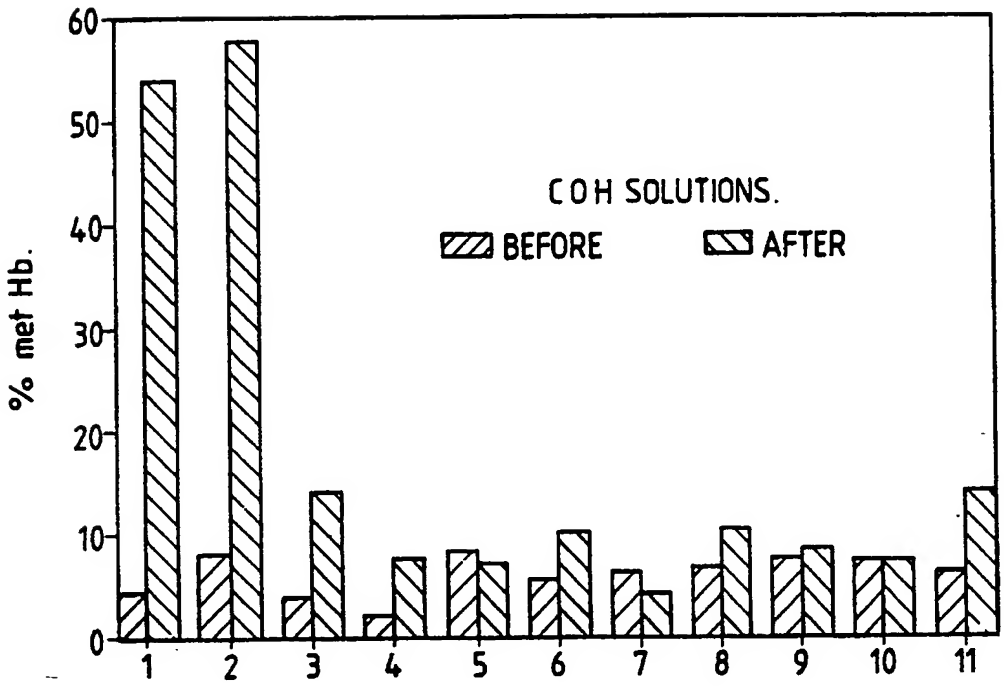
13. A formulation according to claim 9 wherein the polyhydroxy compound is a sugar or related polyols.

14. A formulation according to claim 13 wherein the polyhydroxy compound is selected from trioses, pentoses, hexoses, mono-, di- or oligosaccharides and related polyols.

15. A formulation according to claim 14 wherein the polyhydroxy compound selected from glucose, galactose and non-reducing sugars.

16. A formulation according to claim 15 wherein the polyhydroxy compound is trehalose.

Fig.1.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